Molecular analysis of Rat B cell differentiation.

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7 General discussion and summary

7.1 Introduction

In this thesis we aimed to investigate what stages during rat B cell differentiation selection of the B cell repertoire takes place.

Since many researchers who have studied B cell differentiation, have been using variable nomenclature for the different B cell stages, there is confusion about the different nomenclatures used. By presenting this detailed scheme we hope to provide a more standardized nomenclature and a clearer view on what is presently known about the selective events during B cell differentiation. In the detailed B cell differentiation scheme for the rat (see p.109/110) presented here, the current knowledge about repertoire development during B cell differerentiation is integrated.

7.2 Development of distinguishable repertoires during rat B cell differentiation

The scheme as presented here is a **rat** differentiation scheme although many stages can also be found in the mouse. Whenever a phenotypic marker on a B cell during a particular differentiation stage is specific for the rat, this is indicated by an "R" and when it is specific for the mouse this is indicated by an "M". The different stages presented in this scheme, will be briefly discussed here whereas details about selection processes and factors are discussed in section 7.4 and further.

In the scheme the division into the various B cell differentiation stages was done on the basis of phenotype of the B cells. Most of the phenotypically defined B cell differentiation stages have a distinct repertoire which is described in this scheme in the columns "repertoire" and "definition". On the basis of repertoire, B cell differentiation can be divided into three functionally different phases as originally proposed by Coutinho et al. [1] as a first crude division. The three major types of B cell repertoires which can be distinguished are, *potential, available* and *actual* repertoire.

The *potential* repertoire is determined by the number, structure and mechanisms of expression of the germline genes encoding antibodies. From this *potential* repertoire eventually an *available* repertoire (available for reaction towards antigens) is selected. Finally the *actual* repertoire represents the B cells which upon stimulation by antigens have become plasma cells which are actually secreting antibodies. During the differentiation process from lymphoid stem cell to plasma cell many selectional steps have to be taken which are also indicated in the scheme.

First, the **stem cell** has to become committed to the B cell lineage and then proper rearrangement of the VDJ gene segments has to take place. Hardy et al. [2] have subdivided the $B220^+$, IgM^- B lineage cells in mouse BM cells into four fractions (A, B, C and D) based on differential cell surface expression of determinants recognized by CD43 (leukosialin), BP-1 and HSA [3]. These different phases constitute the stages of progressive VDJ rearrangement (and are

Rat B cell differentiation scheme

Cells	Phenotype	Repertoire	Definition	Origin	Selection process	Selection factors
Lymphoid stem cells	Thy-1$^+$ B220$^-$ Sca$^+$ μ^-	Potential	All genetic possibilities, VDJ-rearr.+ junctional diversity	BM	Commitment to B cell lineage	Growth factors micro-environment (IL-7)
Pro-B cell	Thy-1$^+$ B220$^+$ TdT$^+$ Rag-1/2$^+$ μ-	Potential	All genetic possibilities, VDJ-rearr.+ junctional diversity	BM	Genetic constraints on rearrangement process	Growth factors micro-environment (IL-7)
Pre-B cell	Thy-1$^+$ B220$^+$ TdT$^+$ μ^+ κ^-	Naive, unselected	Realized genetic possibilities before negative selection for (strong) autoAg	BM	Proper rearrangement of heavy chain	V-pre-B, λ5, signaling for correct rearr.
NFB-1	Thy-1$^+$ (R) B220$^+$ IgM^{hi} IgD^{lo}	Naive, selected	Realized genetic possibilities after negative selection for (strong) autoAg	BM	Negative selection for strong auto-Ag	Auto-Ag, concentration, valency, (strength signal regulated by PTP1C). Fas, bcl-2.
NFB-2	Thy-1$^+$ (R) B220$^+$ IgM^{hi} IgD^{lo}	Virgin, available	Available B cells before positive selection by Ag	Periphery (spleen)	Positive selection for useful specificities	(Exogenous) Ag, idiotypes bcl-2

ERF-B (R)	Thy-1^{+} (R) B220^{+} IgM^{lo} IgD^{hi}	Antigen experienced, available	Available B cells after positive selection by Ag	Periphery (a.o. TDL)	Ag-stimulation for GC or PC reaction	Exogenous Ag, idiotypes
RF-B	Thy-1^{-} B220^{+} IgM^{lo} IgD^{hi}	Antigen-experienced, available	Available B cells for primary immune response	Periphery (follicles) lymphocyte corona or mantle zone	Ag-activation for GC or PC reaction	TD-Ag
MZ-B	Thy-1^{-} (R) B220^{+} IgM^{hi} IgD^{lo} HSA^{hi} (M)	Antigen experienced?, available	Available B cells for primary (TI-2) immune response	Marginal zone Spleen	Ag-activation for TI PC reaction	Blood-borne Ag (multivalent?)
Memory B cell	IgG^{+}? IgA^{+}? HSA^{lo} (M)	Antigen-selected, available	B cells (with somatic mutations in their V_H genes) available for secondary immune response	Germinal center Marginal zone? Periphery (site of inflammation)	Ag-selection of higher affinities	TD-Ag Ag on FDC bcl-2
Plasma cell	cyt. Ig+	Actual	B cells actually secreting Ig	medulla spleen + LN, BM	Ag-stimulation to terminal differentiation	Ag (auto-TD,TI)

GC=germinal center, PC=plasmacellular, TD=T cell dependent, TI=T cell independent, FDC=follicular dendritic cell, cyt.=cytoplasmatic, TDL=thoracic duct lymph.

not indicated in the B cell differentiation scheme). Fractions A ($CD43^+$, $BP\text{-}1^-$, HSA^-), B ($CD43^+$, $BP\text{-}1^-$, HSA^+) and C ($CD43^+$, $BP\text{-}1^+$, HSA^+) represent a subdivision of our **Pro-B cells** into three substages: pre-Pro-B, early Pro-B and late Pro-B. In the pre-Pro-B stage there are no D to J joinings yet, which appear in the early Pro-B cell stage and increase in the late Pro-B cell stage. Our **Pre-B cell** stage is equivalent to Hardy's Pre-B cell stage or fraction D in which VDJ rearrangement of the heavy chain is completed as well as to the Pre-B cell stage in Osmonds nomenclature [4]. The selection that can take place here is solely based on the genetic constraints of the VDJ rearrangement process.

The surrogate light chain [5] which is formed by the proteins encoded by $V_{pre\text{-}B}$ and λ-5 can associate with a rearranged heavy chain to be expressed on the membrane of a pre-B cell to provide a signal to proceed with light chain rearrangement. We call this repertoire (before a complete Ig has been constructed), the *naive unselected* repertoire since it has not been in contact with exogenous antigen and no selective processes have taken place yet. Whenever a complete Ig is constructed and expressed on the membrane of the B cell it is called a **newly formed B cell** (NF-B). In the NF-B-1 stage, cells with strong specificity for self are propably eliminated and therefore this stage represents the first selected repertoire (naive selected). The NF-B cells then migrate from the bone mnarrow (BM) to the spleen where they can encounter antigen for the first time which makes their repertoire *antigen experienced.* NF-B cells are probably subject to positive selection between migrating from BM to spleen in which process cells with useful specificities are selected [6]. Kinetic studies have also shown that many cells are lost between the NF-B cells in the BM and in the spleen (from a production of 57 million cells/day in the rat BM to 16 million cells/day in the periphery)[7].

Due to the presence of Thy-1 on rat B cell lineage precursors and a subpopulation of sIg+ B cells precursors, in rat an extra parameter (besides IgM and IgD) is available to distinguish B cell differentiation stages in the rat. Thy-1 is also expressed on a small subpopulation of rat T cells identified as recent thymic emigrants [8]. In mice, Thy-1 is expressed on all thymocytes.

Using the Thy-1 marker in combination with IgM and IgD, four different rat B cell populations can be distinguished [7]. Until now, one of these B cell subpopulations, the **early recirculating follicular (ERF**, phenotype see scheme) **B cells,** has only been characterized in the rat. Furthermore, **NF-B cells** and **marginal zone (MZ) B cells** both are IgM^{hi} IgD^{lo} in mice and rat, but in rat the expression of Thy-1 on the NF-B cells makes them distinguishable from MZ-B cells. Reconstitution experiments [7] have indicated that NF-B cells differentiate to ERF-B cells and finally to **recirculating follicular (RF)-B cells**. Fewer cells die in these late B cell differentiation stages which might suggest that less stringent selective events take place during these stages. MZ-B cells are probably derived from RF-B cells which was observed by several investigators [9-11]. In the most recent experiments by de Boer [11], rats were injected once with adriamycine. Thereafter all their peripheral B cells and all B cell precursors were eliminated, except for RF-B cells. At the moment that RF-B cells were the only B cells present, the marginal

TDL=thoracic duct lymph.

zone already started to repopulate, an event which preceeded the de novo production of NF-B cells.

The **memory B cells** and **plasma cells** may either derive from a common precursor or may originate from separate precursors as already mentioned in the general introduction. Memory B cells are thought to be generated in germinal centers and have accumulated many somatic mutations in their variable region genes. Finally the actual repertoire is represented by plasma cells who are actually secreting antibodies.

7.3 CD5 B cells

In mice, CD5 B cells (B-1 cells)[12] are considered to be a distinct B cell lineage since they show a difference in development pattern, localization, phenotype and function [13-15] in comparison with conventional B cells.

Apart from mice, B-1 cells ($CD5^+$ B cells) have also been found in humans [16] and in rabbits [17], but sofar not in the rat. In chapter 6 we investigated the presence of B-1 cells in the rat. The MRC OX19 antibody was thought to recognize the CD5 antigen in the rat. We confirmed this by cloning the rat CD5 gene and expressing it on COS cells. However, when we used the MRC OX19 antibody to stain rat B cells in various tissues from different rat strains we could not unequivocally reveal by flow cytometry analysis (FCM) the presence of a distinct B cell population expressing more CD5 than other B cells. Possibly B-1 cells are not present in rats or the expression of CD5 is too low to be detected by FCM. Or all rat B-1 cells are B-1b cells, which do not express CD5, but possess all the remaining characteristics of B-1 cells. Alternatively, B-1 cells are present in the rat but can only be characterized by other phenotypic markers. A candidate for the B-1 cell in the rat is still under investigation ($sIgM^{hi}sIgD^{lo}$ Thy-1^+HIS24^{hi}). HIS24 subdivides splenic $sIgM^{hi}sIgD^{lo}$ B cells into two subpopulations: a subpopulation expressing relative high levels of HIS24 and a subpopulation expressing relatively low levels. In spleen sections B cells expressing high levels of the HIS24 determinant are mainly found in the lymphoid follicles [18].

7.4 The potential repertoire of V_H genes is shaped by different evolutionary patterns

The mouse and human V_H gene locus have been studied extensively. V_H gene classification into V_H gene families based on nucleic acid similarity (as originally proposed by Brodeur and Riblet [19]) until now has revealed 14 distinct V_H gene families in mice (reviewed by Kofler [20]) and 7 V_H gene families in humans [21-24]. In chapter 2 we studied the rat V_H gene locus and the presence of 14 distinct rat V_H gene families was established. The 14 rat V_H gene families were named after the homologous mouse V_H gene families (like PC7183, J558, Q52 etc.). In the human the V_H gene families are simply designated V_H1-7.

The three species showed some differences in V_H gene family size. First, the largest family in the rat is PC7183 (19), in the mouse J558 (60) and in the human V_H3 (25)(V_H3 is related to the murine S107, PC7183, J606 and X24 V_H gene families). Second, the rat J558 family (16) and the human J558 counterpart (V_H1)(20) are much smaller than the mouse J558 family (60). Third, the rat X24 family is much larger (18) than the mouse X24 family (2). It is remarkable that the extensive overrepresentation of a single V_H gene familiy (such as the J558 in the mouse) in the genome is not observed in both rat and human.

In inbred mouse strains as well as in wild mice there is considerable restriction fragment length polymorphism [19] whereas the human V_H gene families exhibit surprisingly little polymorphism [25]. In the four investigated rat strains we also observed little polymorphism. From these characteristics (family size and polymorphism) we can conclude that although at the nucleotide level rat V_H genes are more close to murine V_H genes than human V_H genes, rat V_H genes have evolved evolutionary in a similar way as human V_H genes.

The V_H gene families in the mouse show a relatively clustered organization [20] which is in striking contrast to the extensively intermingled organization of the human V_H gene families [22, 23]. The relative order of the rat V_H gene families on the chromosome remains to be elucidated.

To further extend our studies on the rat V_H gene locus we cloned and analyzed 23 germline rat V_H genes as described in chapter 3. Twelve of the germline genes could be qualified as members of the PC7183 V_H gene family, 9 as members of the J558 V_H gene family and 2 as members of the Q52 V_H gene family. We established the evolutionary relationship between the members within the rat PC7183 and J558 V_H gene families and for comparison also for the known members within the homologous mouse V_H gene families. V_H genes represent a typical multigene family and the complete potential repertoire of V_H genes is shaped during evolution by various processes. Some of these processes (gene conversion, homologous but unequal crossing-over) tend to maintain similarity between the genes and others introduce more variation among the genes (gene duplication/divergence). By constructing phylogenetic trees for the different V_H gene families we tried to visualize the evolutionary process within a V_H gene family. From these phylogenetic trees we concluded that the rat J558 V_H gene family had accumulated the most nucleotide changes during evolution. To a lesser extent, evolutionairy nucleotide substitutions were observed in the mouse J558 V_H gene family. The rat and mouse PC7183 V_H gene families accumulated less substitutions and are therefore more conserved than the rat and mouse J558 V_H gene familiy. Gu et al. [6] compared the mouse J558 V_H genes with each other and they were able to define 7 subfamilies. V_H genes which showed more than 90% homology with each other were considered belonging to the same subfamily. In the phylogenetic tree of the mouse J558 V_H gene family the same subfamilies (defined by Gu et al.)[6] could be distinguished on the basis of the branching structure as was to be expected.

Furthermore, this way of dispersion of family members can also be the result of natural selection during evolution, which will influence the likelihood that a change will become fixed

into the genes. The "surviving" V_H genes apparently represent a functionally relevant potential repertoire for survival.

The D gene segments in the mouse have been grouped into three families: D_{Q52} (one member), D_{FL16} (6 members) and D_{SP2} (at least ten members)[26-28]. The human D gene segments can be classified into 7 distinct families (D_{Q52}, D_{LR1}, D_{M1}, D_{N1}, D_{XP1}/$D_{XP'1}$, D_A and D_K)[29-33]. In the rat we found at least 7 different D gene segments of which 3 probably belong to the same family, which shows homology with the mouse D_{FL16} family. Since these D gene segments were obtained from D/J rearrangements (chapter 5) the 3' boundary of the D gene segments is not certain; during D to J joining nucleotides could have been added or removed. However, there was considerable homology between some of the D genes and therefore their 3' site could be established. From the complete VDJ rearrangements (chapter 5), it was not possible to determine the exact germline D segments since both the 5' and 3' boundaries were not certain and the sequences were very heterogeneous and did not show any homology with the D segments from the D/J rearrangements. Rat D segments have not been described before and therefore the data presented here contribute to the knowledge of the potential repertoire of the variable heavy chain of the rat.

In the mouse 4 functional J_H gene segments [34, 35] have been found just like in the rat [36]. The rat and mouse J_H gene segments show high homology with each other(>84%). Since in the human 6 J_H gene segments have been found, Lang et al. [36] suggest that some duplication events must have occurred after the divergence of rodents and primates.

7.5 Mechanisms and factors involved in negative selection

Study of the V_H gene usage in the various differentiation stages can reveal much about the timepoint where selection takes place. However, it does not directly shed any light on the mechanisms of selection or factors involved. On the one hand, selection is aimed at eliminating cells (B and T cells) which are directed against self (negative selection) and on the other hand aimed at building up a useful repertoire (positive selection). Negative selection of selfreactive B cells has been demonstrated in transgenic mouse models[37-40]. Also in T cells, experiments with transgenic mice have shown that those clones which interact too strongly with MHC and self-peptides are eliminated [41-44].

One of the factors involved in negative selection is the cell surface molecule expressed by the Fas gene [45]. The Fas antigen (Ag) is expressed on various T and B cell lines, fibroblast lines, and activated lymphocytes [46, 47] and was originally identified by its ability to trigger cell death in some cell types when crosslinked by antibody [45]. Most of the naturally occurring cell deaths mentioned in this section occur by apoptosis [48, 49], a morphologically defined process including loss of cell volume, membrane blebbing, shrinkage and breakdown of nuclei [50] and

in many cases internucleosomal fragmentation of chromatin [51]. A defect in the Fas gene in autoimmune MRL *lpr/lpr* mice [52] leads to the defective deletion of self-reactive T cells [53]. Since the Fas Ag is also expressed on the surface of B cells, it could also play a role in the selection processes during B cell development (see scheme section 7.2).

The product of the bcl-2 gene has been shown to prolong survival of various hematopoietic cells including B cells [54-58] and T cells [59, 60]. In a study by Itoh et al. [61] the interaction of the Fas Ag and bcl-2 was examined. They showed by transfection experiments that Fas Ag-mediated cell death was retarded and partially inhibited by overexpression of bcl-2 in murine cell lines. Therefore it is likely that regulated expression and interaction of the Fas Ag and bcl-2 determine the fate of T cells [61] and probably also B cells.

Thy-1 also has been found to trigger cell death in immature $CD4^+/CD8^+$ T cells even in bcl-2 transgenic mice who overexpress bcl-2 [62]. Whether Thy-1 also plays a role in B cell cell death is not known. Thy-1 is still expressed on a peripheral rat B cell population (ERF-B cells). If Thy-1 plays a role in inducing apoptosis in B cells then some mechanism prevents that not all Thy-1 expressing cells die. The presence of some regulatory mechanism which represses the death program in mature T cells (still expressing Thy-1) was also suggested by Hueber et al. [62]

Recently, it has been shown that the enzyme protein tyrosine phosphatase 1C (PTP1C) which is widely expressed in hemopoietic cells, is an important negative regulator of antigen receptor signaling and selection in B lymphocytes [63]. Signaling by cell surface antigen receptors coordinates development and selection of B and T lymphocytes. The thresholds of quantitative differences in antigen receptor signaling are determined by negative regulation through PTP1C. Mice with a defect in PTP1C (motheaten (me)[64] and motheaten viable (me^v) mice [65]) lack mature conventional B cells and make poor antibody responses following immunization. Negative selection is too strong and not properly downregulated by PTP1C in these mice and therefore they lack mature conventional B cells. PTP1C may inhibit the activity of some kinases which are intermediates in transmitting intracellular signals from B and T cell antigen receptors towards activating downstream signaling cascades [66, 67] leading eventually to cell death.

Another characteristic of me^v mice is the presence of a distinct population of B-1 cells in contrast to the deficiency of conventional B cells [68]. Me^v mice develop autoantibodies by 4 weeks of age probably produced by B-1 cells [69, 70]. In contrast to conventional B cells, B-1 cells apparently escape the increased stringency of negative selection caused by the defective PTP1C enzyme [63]. In a transgenic model of autoreactive B cells specific for an erythrocyte surface autoantigen, it was also found that B-1 cells escape negative selection [71]. Since B-1 cells are a major source of autoantibodies and therefore probably also involved in many autoimmune diseases, elucidation of the escape mechanism by B cells from negative selection is of crucial importance. In a study by Mercolino et al. [72] it was observed that anti-bromelain mouse red blood cells (BrMRBC) increased with age during normal ontogeny. Anti-BrMRBC reactive antibodies are almost exclusively produced by B-1 cells [13, 73]. Cells with the anti-

BrMRBC specificity (B-1 cells) probably are positively selected by the availabilty of antigen as suggested by Hardy et al. [74].

7.6 Mechanisms involved in positive selection

So far the factors involved in positive selection for B cells are less clear than those involved in negative selection. For T cells it is known that positive selection takes place in the thymus whereby especially the cortical thymic epithelial cells play a role in this process [75]. The T cell receptor (TCR) repertoire must be shaped in such a way that it is capable of recognizing and responding to myriad foreign antigens in the context of self-MHC. Positive selection in the T cells takes place to ensure that receptors which have at least some recognition of self-MHC are selected. In B cells it is thought that foreign antigens and /or endogenous ligands, like antibodies play a role in positive selection. In a study by Freitas et al. [76] the V_H gene family usage was investigated in the BM (naive (un)selected) and splenic (available) repertoire of conventional (SPF) and germfree mice. The V_H gene family usage in BM of conventional mice was similar to the usage in the BM of germfree mice. The V_H gene family usage in spleen of conventional mice differed from the usage in the BM, whereas the V_H gene family usage in the germfree spleen was similar to the usage in the BM. These results suggest that environmental factors may influence the expressed B cell repertoire by selecting B cells expressing certain V_H gene families as was also already suggested by other researchers [77, 78]. Administration of serum immunoglobulins from conventional adult mice to germfree mice resulted in the same V_H gene family usage as in normal conventional mice. This shows that the introduction of a large panel of foreign molecules (IgG) can have an effect on the selection process either directly by stimulation of antigen specific clones or indirectly by idiotypic interactions. The regulatory role of an idiotypic network for the immune system was already theorized by Jerne [79]. The fact that a small amount of anti-idiotypic antibodies can influence the repertoire of the B cells during ontogeny was shown by Kearney and Vakil [80].

7.7 Peripheral Selection

Selection can take place in the periphery as was demonstrated by Russel et al. [40]. Negative selection might be necessary to delete auto-antibodies in the post BM state to prevent organ-specific autoimmunity. In chapters 4 and 5 we investigated the repertoire of two peripheral B cell populations, representing two distinguishable B cell differentiation stages. The repertoire of the ERF-B and RF-B cells, an available and antigen experienced repertoire, was studied by the determination of the usage of V_H gene families. Our results suggest that both the ERF-B and RF-B cell populations are probably already positively selected populations since the V_H gene family usage was not according to the size of the rat V_H gene families.

Between the ERF-B cell population and the RF-B cell population no significant difference in V_H gene family usage was observed. This suggests that between these stages there is no selection at least on the basis of V_H gene family usage. This finding was supported by kinetic studies [7] in which hardly any cell loss was observed between the ER-B cell stage and the RF-B cell stage. However, a closer look at the individual V_H genes within both populations revealed that none of the V_H genes in the ERF-B cell population were used in the RF-B cell population. Within the PC7183 V_H gene family, none of the V_H genes found in the ERF-B cell population has been found in the RF-B cell population. Since some of the V_H genes are overrepresented in the RF-B cell population (as was also observed by Gu et al. [6] in comparing the repertoires of NF-B cells and RF-B cells in mice) some positive selection may have occurred between the ERF-B and RF-B cell stage. This suggests that selection is an ongoing process during late B cell differentiation stages. Determination of the V_H gene usage of MZ-B cells might provide more insight into their origin and characteristics. MZ-B cells can be found in a compartment (marginal zone) which is only present in the spleen as a broad band around B cell follicles. MZ-B cells are thought to develop from RF-B cells and most of the B cells in the MZ are probably virgin B cells [11, 81]. This need not imply that all RF-B cells end up in MZ-B cells: analysis of V_H gene usage will show whether only few RF-B cells are positively selected to transform into MZ-B cells. In addition there are also indications for the presence of memory B cells in the MZ [82, 83].

7.8 Final conclusions

In conclusion, the B cell repertoire undergoes many selective events:

First, the composition of the potential repertoire of the B cell is shaped by selective events during evolution.

Second, the transgenic mouse models have provided strong evidence for the occurrence of negative selection during B cell differentiation. Formally, negative selection has not been clearly proven in normal non-transgenic mice. Evidence for positive selection originates from experiments in normal mice and rats, which occurs at all tested peripheral B cell differentiaton stages.

Finally the repertoire is fine tuned to its environment by antigen selection of somatically diversified B cells.

In our view, selection of B cell repertoires is probably a continuous process which starts in the BM with sIg^+ B cells and continues until the production of plasma cells.

Insight into these selective processes might lead to a better understanding of e.g. autoimmune diseases and to rationalization of vaccine programs to prevent infectious diseases.